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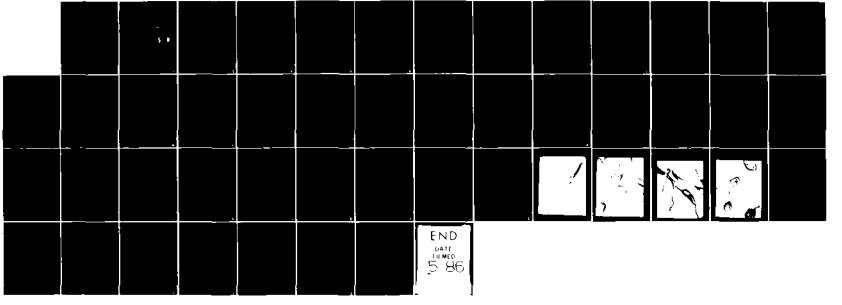

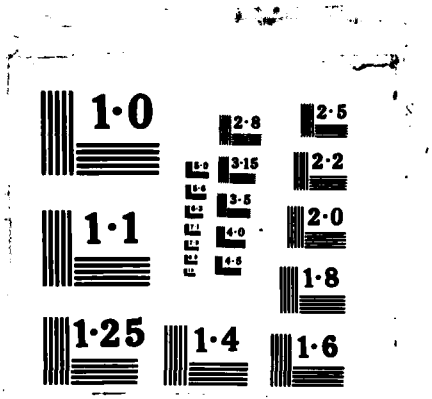
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Biochemistry of Trypanosomatidae of Importance in Africa

Annual Report

LINDA L. NOLAN, Ph.D.

DECEMBER 1983

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701 - 5012

Contract No. DAMD17-81-C-1198

University of Massachusetts
Amherst, Massachusetts 01003

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to synthesize purines de novo and consequently are obligate salvagers of preformed purines.

This project involves an investigation of purine and pyrimidine metabolism of Leishmania. Comparisons of their biochemistry will be made within the

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parasitic group and to their host cells.

Basic information regarding metabolic capacities of these organisms will be obtained. Attention will be given to the mechanisms by which these organisms absorb nutrients from their environment. These mechanisms involve enzymes excreted into their surroundings, enzymes located on the cell surface, and enzymes located within the cell.

Enzyme and transport mechanisms which exhibit differences from those of host cells will offer targets for chemotherapeutic exploitation. Inhibitors will be sought which will affect these target systems. Those inhibitors which are leishmanicides will then be tested in an appropriate animal system.

Preliminary studies have shown that allopurinolriboside and formycin B appear to be effective analogues against certain species of leishmania and trypanosomas. The biochemical mode of action of these compounds appears to be similar but to vary quantitatively. Our preliminary results suggest that the most critical action of these drugs occurs by their interaction with RNA and DNA. Investigations are being made on the mode of action these compounds have on the RNA and DNA of sensitive organisms, and how this process can be reversed by the addition of other purines.

Other purine and pyrimidine analogues found to be inhibitory to leishmania will be investigated for their effect on interference with nucleic acids and their metabolism.

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SUMMARY

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to synthesize purines de novo and consequently are obligate salvagers of performed purines.

This project involves an investigation of purine and pyrimidine metabolism of Leishmania. Comparisons of their biochemistry will be made within the parasitic group and to their host cells.

Basic information regarding metabolic capacities of these organisms will be obtained. Attention will be given to the mechanisms by which these organisms absorb nutrients from their environment. These mechanisms involve enzymes excreted into their surroundings, enzymes located on the cell surface, and enzymes located within the cell.

Enzyme and transport mechanisms which exhibit differences from those of host cells will offer targets for chemotherapeutic exploitation. Inhibitors will be sought which will affect these target systems. Those inhibitors which are leishmanicides will then be tested in an appropriate animal system.

During the period of this contract, uptake capabilities of L. donovani (Khartoum strain-drug sensitive visceral leishmaniasis) and L. mexicana panamensis WR 227 were investigated. In L. donovani WR 130 it was found that N^6 Methylaminopurine inhibited uptake of hypoxanthine, guanine and to a lesser extent adenine. 6-Methylaminopurine 9-ribofuranoside inhibited uptake of adenosine and to a lesser extent guanosine.

In L. mexicana WR 227 it was determined that at least two loci exist for the transport of nucleosides, one for adenosine and one for inosine and guanosine.

Uptake experiments using ^{3}H-formycin B, showed that its uptake is inhibited by inosine and guanosine, but not by adenosine.

The following enzymes in L. mexicana were tested for inhibition by formycin B (2 μM - 1 mM) with no significant effect observed.

- adenine, guanine and hypoxanthine phosphoribosyltransferases
- Guanase
- Adenase
- Inosine, guanosine and adenosine nucleosidases

Preliminary studies have shown that allopurinolriboside and formycin B appear to be effective analogues against certain species of leishmania and trypanosomes. The biochemical mode of action of these compounds appears to be similar but to vary quantitatively. Our preliminary results suggest that the most critical action of these drugs occurs by their interaction with RNA and DNA metabolism. Investigations will be made on the mode of action these compounds have on the RNA and DNA of sensitive organisms, and how this process can be reversed by the addition of other purines. The information gained from these studies will be useful in the design of more potent and specific chemotherapeutic agents.

Other purine and pyrimidine analogs found to be inhibitory to leishmania will be investigated for their effect on interference with nucleic acids and protein metabolism.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

METHODS

Routine Methods: more specific procedures currently being used in our laboratory are disussed in the section entitled "Previous Work on This Project."

Culture Methods:

The organisms used in this project have been obtained from the Walter Reed Army Institute of Research through the courtesy of Dr. Joan Decker-Jackson and Dr. Jonathan Berman. The organisms used most have been Leishmania mexicana panamensis WR 227 and L. donovani WR 130 (Khartoum strain drug sensitve visceral leishmaniasis). Other organisms presently being cultivated in this laboratory are L. braziliensis WR 424 (Murray isolate from Panama causing cutaneous leishmaniasis), L. braziliensis WR 063 (Terborgh isolate from Peru, causing mucocutaneous leishmaniasis). These organisms are maintained by weekly transfers into Schneider's medium [Grand Island Biological Co., Grand Island, N.Y. (Gibco)] containing 10% heat inactivated fetal bovine serum (HIFBS: GIBCO).

For growing large batches of leishmaniae promastigotes, Brain Heart Infusion Medium (BHI) containing 37 g Difco Brain Heart Infusion/liter water, 10% heat inactivated serum and 26 µg hemin/ml is used. Cells are grown at 26° C in 2000 ml wide Fernbach flasks containing 250 ml of BHI and harvested during the exponential growth phase (∿ day 4).

For defined biochemical experiments we use the media of Steiger and Black. We use this media for all transport, uptake and reversal experiments. To deplete cells of purines we transfer an inoculum from Brain Heart Infusion into Steiger and Black minus purine, but with 5% serum, and incubate these cells 48 hr at 26°C. We then aseptically centrifuge these cells 500 x g for 10 min. and resuspend them into fresh Steiger and Black minus purine. We resuspend to the desired number of cells and then add the purine or analogue we wish to test. By treating the cells in this manner, we avoid as much as possible interference of the metabolism of the compound being tested by purines in the media.

RE 1X (Steiger and Black)

Components per liter:

A)
- 8.0g NaCl
- 400 mg KCl
- 200 mg $MgSO_4 \cdot 7H_2O$
- 60 mg $Na_2HPO_4 \cdot 2H_2O$
- 60 mg KH_2PO_4
- 2.0g glucose

B)
- 200 mg L-arginine
- 100 mg L-histidine
- 100 mg L-isoleucine
- 300 mg L-leucine
- 250 mg L-lysine.HCl
- 50 mg L-methinonine
- 100 mg L-phenylalanine
- 300 mg L-proline
- 400 mg L-threonine
- 50 mg L-tryptophan
- 50 mg L-tyrosine
- 100 mg L-valine

C)
- 300 mg L-glutamine
- 1.0 g $NaHCO_3$
- 14.25 g HEPES (=60mM)
- 20 mg adenosine

D)
- 1 mg D-biotin
- 1 mg choline chloride
- 1 mg folic acid
- 2 mg i-inositol
- 1 mg niacinamide
- 1 mg D-pantothenic acid (hemi-calcium salt)
- 1 mg pyridoxal. HCl
- 0.1 mg riboflavine
- 1 mg thiamine. HCl

E)
- 2.5 mg haemin

L. donovani will be maintained in male Syrian hamsters by intraperitoneal injection of infected hamster spleen homogenate. Amastigotes will be isolated by sterile removal of infected spleens which are then homogenized and the parasites cleaned by differential centrifugation (Krassner, 1966).

Enzyme Studies

In all cases where it becomes critical to resort to enzyme purifications the sequence of steps envisioned are gel filtration, isoelectrofocusing, column chromatography, gel electrophoresis, various pH changes and heat treatments. Activities will be largely determined by the use of radioactive substrates. When possible, commercial mammalian enzyme preparations will be used to compare with enzymes being studied in the protozoans; otherwise, mammalian (bovine and/or rat) liver will be used as a source of tissue.

Enzyme activities are determined by paper chromatography or by paper electrophoresis as has been previously described (Kidder, Dewey and Nolan, 1977), following incubations of reactants with [^{14}C] - labeled substrate. When inhibitors are used they are added to the enzyme-buffer solution, at optimum pH, and preincubated 10 min at 35^{o}. After reaching equilibrium at 35^{o} the radioactive substrate is added. The reaction is stopped at the appropriate time and the reaction mixture is streaked on on Whatman #1 paper for descending chromatography or for electrophoretic separation. Radioactive peaks are located with the aid of a Tracerlab 4π scanner and identified by co-chromatographs of authentic samples. Quantification is accomplished by determining the areas under the peaks by planimetry. Some enzyme activities are determined using the HPLC equipped with a scintillation counter.

In those cases where radioactive substrates are not commercially available, they will be synthesized enzmatically from an available radioactive precursor. Spectrophotometric assays will be carried out if a radioactive assay is not feasible. In general the assay methods that will be used are those described in Methods of Enzymology LI, Purine and Pyrimidine Metabolism, 1978.

Transport and Accumulation Studies

These studies are carried out using whole cells of the parasites along with labeled purine and pyrimidine bases and nucleosides. Incubations are carried out in 1.5 ml microcentrifuge tubes in the presence and absence of substrates and/or compounds being tested for inhibition.

By transport is meant the events essential to translocation of the substrate across the cytoplasmic membrane. As a consequence of this translocation, substrates may become available to intracellular enzymes and metabolized to chemical forms that are not substrates for the transport systems. The accumulation of such metabolites (as well as of the chemically unaltered substrate) is properly referred to an uptake. Uptake is several steps removed from transport. The availability of energy, which may determine the concentration of substrates against electrochemical gradients and the activities of intracellular enzymes which affect their metabolism, may limit uptake but are not directly related to the transport event (Berlin and Oliver, 1975).

The most serious problem in studying the process of transport as distinct from uptake is the failure to determine rates at sufficiently early times. As with enzyme reations, it is essential to measure initial rates in order to determine unidirectional flux. This period is clearly incompatible with prolonged washings or centrifugations, and rapid sampling methods must be employed. A technique was devised by Kidder, Dewey and Nolan (1978)which facilitates rapid measurement. This procedure is based on rapid separation of medium from cells by centrifugation. Aliquots of cell suspension are placed in plastic centrifuge tubes (1.5 ml capacity), radioactive substrate added, vortexed briefly and the tubes placed in an Eppendorf microcentrifuge, and at 5 sec. the cells are rapidly sedimented. Aliquots are placed in vials for counting. The cells are then resuspended by vigorous vortexing and two further samples are taken to obtain total (zero time) counts. The presence of cells in the latter samples necessitate a correction for self absorption. The difference between the counts represents the amount of substrate taken in by the cells.

The factor used for correction of self absorption by whole cells is obtained by incubating cells with AMP (P^{32} labels, which does not encter C. fasciculata) spinning them down and taking aliquots of the supernant, then resuspending the pellet by vortexing and determing radioactivities. The difference between counts obtained with supernant plus cells and supernant is taken as self absorption. Thirty duplicate experiments are conducted and an average taken.

This technique that we have used for C. fasciculata has been successfully used in investigating uptake of purine bases and nucleosides in African trypanosomes (James and Born, 1980).

Transport and uptake experiments dealing with macrophage and tissue culture cells will be by the method of Berlin and Oliver (1975).

Protein

Protein will be estimated by methods II, III or IV of Layne (1957) or by the method of Kalb and Bernlohr (1975), depending on the amount of protein and nucleic acids in the sample.

Inhibitor Studies

Most inhibitors selected for testing will be structural analogs of the enzymes or transport systems substrate or product. Compounds which appear as likely cnadidates include the following:

Formycin B
Formycin-B-monophosphate
Formycin-A-monophosphate
Formycin-A-diphosphate
Formycin-A-triphosphate
Allopurinol
Allopurinolriboside
4-amino-(3, 4-d) pyrazolopyrimidine 6-4, - Dimethylallylaminopurine

phenylhydrazinopyrimidine
hydroxyphenylazopyrimidine
2-Aminopyrimidine
N6-Methyladenine
N6-Dimenthyladenine

6-Mercaptopurine
6-Methylmercaptopurine
6-Chloropurine
2-Methyladenine
1-Deazaadenine
Purine
2,6-Diaminopurine
2-Azaadenine
2-Aminopurine

Isoguanine
8-azaguanine

2-Hydroxypurine
8-Methyladenine
2-Methylthio-6-aminopurine
Kinetin
N1-Methylhypoxanthine
6-Methyl-2-oxypurine
6-Phenylaminopurine
6-Benzylaminopurine
6-Bromopurine
6-Methoxypurine

coformycin
deoxycoformycin
erythro-9-(2-hydroxy-3-nonyl) adenine
6-mercapto-9-(tetrahydro-2-furyl)-purine
2,6-bis-(hydroxyamino)-9-B-D-ribofuranosyl-purine
6-iodo-9-(tetrahydro-2-furyl)-purine
Xylosyladenine
5-methylrotate
isoocrotic acid
4,2-substitutad, oxazolo-[5,4-d]-pyrimidine -7-one
3-amino-4-carbethoxypyrazole
3-amino-4-carboxypyrazole

N^6-benzylaminopurine
N^6_6-(2-naphthylamino) purine
N^6_6-(2-thenylamino) purine
N^6-(2-pyridylamino) purine
5-cyanouracil
5-iodouracil
5-fluorocytosine
Azapyrimidines
5-fluorouracil
6-aminothymine
dihydroazaorotate
5-nitroorotate

isoorotic acid

In the event that derivatives of formycin are synthesized by Dr. Roland K. Robins, Director of the Cancer Research Center in Provo, Utah, these will be tested for growth inhibition and if effective their mode of action will be determined at the membrane, enzyme and RNA & DNA level. Dr. R.K. Robins is under contract with WRAIR.

Phase Contract Photography

Compounds which affect purine or pyrimidine metabolism will be investigated to see what effect they have on cellular morphology. These studies will be carried out with the use of a Zeiss phase contrast microscope equipped with a polaroid land camera.

Metabolic Fate of Inhibitors

Spectroscopic and chromatographic methods will be used to determine the metabolic fate of inhibitors.

Most of the metabolism studies will be performed using our Waters HPLC system equipped with a UV-detector and an on-line scintillation counter (HS model) purchased from Radiomatic Instruments. Using this system we are able to use dual labeling, which allows us to follow the radioactive fate of two radioisotopes at one time. Depending on the type of separation (anion-exchange or reversed-phase), the system measures simultaneously the concentration and radioactivity for a given chromatographic peak. Rapid and reproducible results may be obtained and recorded as both analog (peak trace) and digital output (peak integration). Through the use of purine and pyrimidine precursors, it is possible to study in a systematic way the overall metabolism of nucleotides, nucleosides and bases

Our system is similar to that of H. Kyle Webster's at WRAIR.

At the present time we are using a radial-pak μ Bondpak C-18 cartridge with a Z-module system (Waters Assoc., Milford, MA) for separation of purine bases and nucleosides. An isociatic solvent of 50% methanol: H_2O is being used to separate most bases and nucleosides, but to separate formycin A from formycin B we use 10% methanol: H_2O at a flow rate of 3 ml/min. (Fig. 1). To separate nucleosides and nucleotides, we use a Waters Radial-Pak SAX cartridge with the Z-module using a gradient of KH_2PO_4:KCl and varying flow rates (Fig.A,B). As regards the analysis of formycin B and its metabolites we are trying the conditions reported by Rainey & Santi (PNAS, 1983, February issue) and of Rossomando, et al (Anal. Biochem. 116, 80-88, 1981). The procedure used by Rossomando takes advantage of the fluorescent properties of the formycin derivatives which is much more sensitive than UV-detection. He also uses a reverse-phase isocratic system for separation of formycin nucleotides which is quicker and has less UV background noise than the conditions reported by Rainey & Santi.

Preparation of cell extracts are done by the method of Pogolotti & Santi (Anal. Biochem. 126, 334-345, 1982). Briefly, this method involves 0.6MTCA extraction and centrifugation at 4°C and the supernant is aspirated and added to an equal volume of cold freon containing 0.5 M tri-n-octylamine. The mixture is vortexed and centrifuged (12,000 g, 30 sec.) at 4° C, the lower phase is removed by aspiration leaving the near neutral aqueous solution of nucleotides. These extracts are then stored at -70°C if not used immediately.

A) 10% Methanol-H_2O	Isocratic Elution	Time	Flow	% A	% B	Curve
		Initial	3	100	0	*

chart speed: 0.5 cm/min

column: Radial-PAK μBONDAPAK C-18 Cartridge, 8mm X 10 cm with Z-Module System

peaks: #1 Formycin B
#2 Formycin A

sample: 0.5 mM Formycin A
0.5 mM Formycin B

absorbance: 254 nm

sensitivity: 0.05

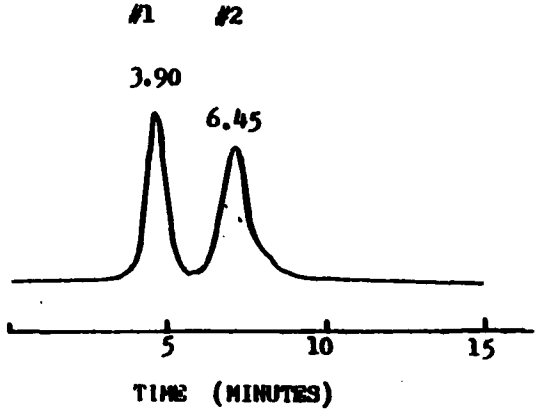


Fig. 1 Separation of Formycin Compounds

Partial Purification of RNA Polymerase II

The DNA-dependent RNA polymerase II or B from the lower eukaryote Physarum polycephalum has been purified to apparent homogeneity by a new method employing poly (ethylene imine) precipitation and elution, and heparin-Sepharose affinity chromatography (Smith and Braun, 1978). We are presently modifying their method of purification for isolation of RNA polymerase from Leishmania.

The following column and conditions will be used in the purification procedure.

Buffers

Buffer 1 will contain 50mM Tris pH 7.9, 25% (v/v) glycerol, 0.1mM EDTA, and 10mM 2-mercaptoethanol. Buffer 2 is buffer 1 only containing 5mM $MgCl_2$. Buffer 3 will contain 50mM Tris pH 7.9, 25% (v/v) glycerol, 0.1MM EDTA, 1mM dithiothreitol.

Poly (ethylene imine)

A 10% (v/v) solution of poly (ethylene imine), will be prepared and neutralized.

DEAE- Sepharose

DEAE-Sepharose CL-6B will be used as supplied from Pharmacia Fine Chemicals after equilibration with buffer 2 containing 0.18M $(NH_4)_2SO_4$.

Heparin-Sepharose

Separin-Sepharose will be prepared by the method of Iverus (1971) using CNBr-activated Sepharose 4B obtained from Pharmacia Fine Chemicals. Heparin will be reacted with the CNBr-activated Sepharose at a concentration of 1.43mg heparin/ml swollen gel.

Phosphocellulose

Whatman P-11 phosphocellulose will be washed with acid and base as described by Burges, R (1969) and equilibrated with at least 25 column volumes of buffer 1 containing 0.05M $(NH_4)_2SO_4$ before use.

RNA Polymerase Assay

The 'low UTP' RNA polymerase assay used in purification assays will be modified from that described by Burgess and Burgess (1974). It will have a final volume of 0.2 ml and will contain: 1mM dithiothreitol, 1mM $MnCl_2$, 5mM $MgCl_2$, 50mM Tris pH 7.9, 10% (v/v) glycerol, 100ug/ml bovine serum albumin, 30ug/ml denatured calf thymus DNA, 0.6mM each ATP, CTP, GTP and 0.5uM [^{3}H] UTP (41 Ci/mmol). For the purpose of determining the enzyme activity in the pooled fractions at the various stages in the purification, a 'high UTP' assay mixture will be employed. This reaction mixture is identical to the low UTP assay mixture except that the $MgCl_2$ is omitted, the total UTP concentration is raised to 0.6mM, and enough [^{3}H] UTP is added to bring the final specific radioactivity of [^{3}U] UTP to 0.413 Ci/mmol.

All reaction mixtures will be terminated with 5% (w/v) trichloroacetic acid containing 5mM $Na_4P_2O_7$. The trichloroacetic-acid-insoluble material will be collected on glass fiber filter discs (Whatman GF/C) and washed thoroughly with 2% (w/v) trichloroacetic acid containing 5mM $Na_4P_2O_7$. After washing the filter discs once in ethanol, and drying them at 80°C for 5 min, they will be counted in a scintillation mixture. A unit of RNA polymerase activity will be defined as that amount of enzyme activity which will catalyse the incorporation of 1 nmol of UMP into trichloroacetic-acid-insoluble material in the high UTP assay mixture in 10 min under the conditions stated.

Partial Purification of DNA Polymerase

Cells are sonicated in 0.1M Tris-HCl, pH 7.8 buffer with 20% (v/v) glycerol and fractionated by centrifugation at 4° in a Sorvall SS-34 rotor at 10,000 x g for 1 hr. The pellet is discarded, and the resulting supernatant fraction is made 1% (v/v) in respect to Triton X-100 and 0.4M in respect to KCl. After gentle mixing the solution is centrifuged in a Beckman SW41 rotor at 100,000 x g for 45 min. at 4°. The supernatant fraction is then passed through a DEAE-Sephadex column equilibrated with buffer containing 0.05M KCl, 1mM dithiothreitol, 0.4M KCl and 10% glycerol to remove any associated nucleic acids. The peak fractions are pooled, concentrated by ammonium sulfate, dialyzed and put on a phosphocellulose column equilibrated with 50mM KCl (Buffer A). The column is then washed with 20ml of buffer containing 70mM KCl and eluted with a 200ml linear KCl gradient (0.1 - 0.6 MKCl) in 50mM Tris-HCl, pH 7.5, 10% glycerol.

The active fractions from this column are then passed over a DNA-agarose affinity column ($0.8cm^2$ x 15cm) at 0.15M KCl, and eluted with 120ml linear gradient of 0.15 M - 1.2M KCl in 0.1M Tris - HCl, pH 7.8 buffer.

DNA Polymerase Assay

A procedure developed by Sigma Chemical Co. is used in our laboratory. The procedure involves the use of a pyrophosphate reagent which is useful in measuring activity of enzymes which generate PPi. Described below is Sigma's non-radioactive assay for DNA polymerase using the pyrophosphate assay reagent.

PRINCIPLE:

$$d(pN)_x + dNTP \xrightarrow{\text{DNA polymerase}} d(pN)_{x+1} + PP_i$$

$$PP_i + F\text{-}6\text{-}P \xrightarrow{PP_i\text{-}PFK} F\text{-}1,6\text{-}DP + P_i$$

$$F\text{-}1,6\text{-}DP \xrightarrow{\text{Aldolase}} GAP + DHAP$$

$$GAP \xrightarrow{TPI} DHAP$$

$$2DHAP + 2\beta\text{-}NADH + 2H^+ \xrightarrow{GDH} 2 \text{ glycerol-3-phosphate} + 2\beta\text{-}NAD^+$$

Abbreviations: d(pN) = Deoxypolynucleotide
dNTP = Deoxynucleoside triphosphate
dATP = Deoxyadenosine triphosphate
dCTP = Deoxycytidine triphosphate
dGTP = Deoxyguanosine triphosphate
TTP = Thymidine triphosphate

REAGENTS INCLUDE:

A. 600 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 10 mM Mercaptoethanol.

B. 1 mg/ml activated calf-thymus DNA.

C. 2 mM each dATP, dCTP, dGTP.

D. Pyrophosphate Assay Reagent reconstituted with 4 ml of water.

E. 8% Ampholytes, pH 3-10.

F. 1.0 mM pyrophosphate standard.

G. 2 mM TTP.

UNIT DEFINITION:

One unit of DNA polymerase will generate 10 nanomoles of PP_i in 30 minutes at 37°C.

CALCULATION:

$$\text{Units/cuvet} = \frac{A_{340}/\text{min} \times 3 \text{ ml} \times 30 \text{ min}}{6.22 \times 2^{*} \times 0.01\mu\text{moles}}$$

* The factor 2 accounts for the fact that for 1 mole of pyrophosphate consumed there are 2 moles of β-NADH oxidized.

Isolation of Ribosomal Proteins

There appears to be very little information on the isolation of ribosomal proteins in Leishmania, so we will use the method reported by Petridou, Cuny and Hayes (1983) for the isolation of ribosomal proteins from Tetrahymena.

This method involves all operations to be carried out at 0-4°. Cells will be harvested by centrifugation (2000 x g, 10 min), washed by centrifugation in a solution containing 20 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 0.25M sucrose, pH 7.6, and resuspended at a density of 10^7-10^8 cells/ml in the same buffer with or without addition of 5 mM iodoacetamide. Nonidet P_{40} will then be added to a final concentration of 0.24% (w/v), and lysis will be allowed to proceed for 10 min in an ice bath. The lysate will then be centrifuged at 12,000 x g for 15 min. The clear supernatant will be recovered, and its composition adjusted to 20 mM Tris-HCl, 250 mM KCl, 2.5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, pH 7.6.

Ribosomal subunits in the final suspension will be separated by centrifugation on 35-ml 10-30% sucrose gradients prepared in 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 250 mM KCl, 6 mM 2-mercaptoethanol, pH 7.6. Gradients will be loaded with 2-3 ml

of suspension (100-150 A_{260} nm) and centrifuged at 20,000 rpm for 16 hr (SW 27 rotor). Subunits will be concentrated from pooled gradient fractions by centrifugation at 80,000 g for 20 h, after raising the Mg^{++} concentration to 4mM, or by precipitation with polyethylene glycol (final concentration 12% [w/v]) at -20°C for 20 min after raising the Mg^{++} concentration to 10mM. Precipitatated subunits will be collected by centrifugation at 12,000 x g for 15 min. Subunit pellets prepared by either method will be resuspended in 20 mM Tris-HCl, 240 mM KCl, 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, at a concentration of 100-200 A_{260} nm/ml and stored at -70°.

Protein samples for one-dimensional gel electrophoresis will be prepared by incubating suspensions of 40S (0.72A_{260}) or 60S (0.95 A_{260}) subunits in 50µl of 0.05 M Tris-HCl, pH 6.8 SDS 2% (w/v), glycerol 7.5% (v/v), 2-mercaptoethanol 1% (v/v), at 100° for 3 min. Samples will be cooled and the 2-mercaptoethanol concentration raised to 6% (v/v) before loading onto gels. Polyacrylamide gel electrophoresis of ribosomal proteins will be carried out using one-dimensional SDS-polyacrylamide gel electrophoresis as described by Adoutte Panvier (1980). We will also try to develop a method of separating ribosomes by HPLC in collaboration with Dr. Lallan Giri at Pharmacia Fine Chemical Co.

Assay of Ribosome Function

To assay in vitro reassociation of ribosomal subunits, samples of purified 40S and 60S particles will be dissolved together at 0°C in 150µl of 20mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, pH 7.6. The solutions will then be incubated at 28°C for 30 min, cooled to 0°C, and 100-µl samples will be analyzed by sedimentation at 19,000 rpm for 16 h at 4°C on 3.6ml 10-30% (w/v) linear sucrose gradients prepared in 20 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, 6 mM 2-mercaptoethanal. The distribution of A_{260} nm absorbing material in the centrifuged gradients will be recorded and the percentage of this material present in the form of 80S ribosomes will be calculated.

Poly U-directed polyphenlalanine synthesis and peptidyl transferase activity will be measured as described by Rodrigues Pousada and Hayes (1976).

Leishmania Amastigotes in a Cell-free Medium

A modification of the medium developed by (Pan, 1984 Experimental Parasitiology) will be used to investigate the purine metabolism of L. mexicana pifanoi, an organism which was originally isolated from a skin lesion of a Venezuelan patient suffering from disseminated cutaneous leishmaniasis and sent to H.S.P.H. by Dr. J. Convit in 1960 (Convit, Kerdel-Vegas and Gordon, 1962). The strain was cloned by Pan to obtain a genetically homogeneous population. The media developed by Pan are modifications of F-69 (Pan, S.C., 1978) for the cultivation of Trypanosoma cruzi amastigotes. The major modifications are: (1) the final concentration of fetal bovine serum was raised to 25%; (2) HEPES was used as a buffer; (3) the concentration of sodium bicarbonate was increased;

and (4) a higher concentration of nucleotides was used in the stock solution.

For our investigations we will vary the purine concentrations in this medium to observe the effect on promastigate to amastigate transformation. We will also use different radiolabelled purines to investigate their uptake and metabolism during the transformation process.

PREVIOUS WORK ON THIS PROJECT

Efforts during the last two years have centered on the following areas:

- Characterization of purine and pyrimidine transport and uptake capabilities, and testing of analogues to inhibit these pocesses.
- The testing of purine and pyrimidine analogues in growth experiments using a biochemically defined medium.
- The setting up of a high performance liquid chromatography system equipped with a UV-detector and an on-line scintillation counter. Personnel have been trained at the Water's HPLC school in Milford, MA. Techniques for the separation of purine and pyrimidines are being tested to determine which ones best suit our needs.
- The metabolism and mode of action of formycin B is being investigated and being compared to that of allopurinolriboside and 4-aminopyrazolopyrimidine at the (a) growth level, (b) enzymatic level and (c) at the level of translation.

Research during the next two years will be a continuation of all the above with the major effort in comparing the mode of action of formycin B to that of allopurinolriboside and 4-aminopyrazolo (3,4-d) pyrimidine at the enzymatic level and at the level of translation and transcription.

$$\text{DNA} \xrightarrow[\text{transcription}]{} \text{RNA} \xrightarrow[\text{translation}]{} \text{protein}$$

During the period of this contact, uptake capabilities of L. donovani (Khartoum strain-durg sensitive visceral leishmaniasis) and L. mexicana panamensis WR 227 were investigated. In L. donovani WR 130 it was found that N^6 Methylaminopurine inhibited uptake of hypoxanthine, guanine and to a lesser extent adenine. 6-Methylaminopurine 9-ribofuranoside inhibited uptake of adenosine and to a lesser extent guanosine.

In L. mexicana WR 227 it was determined that at least two loci exist for the transport of nucleosides, one for adenosine and one for inosine and guanosine.

Uptake experiments using ^{3}H-formycin B, showed that its uptake is inhibited by inosine and guanosine, but not be adenosine.

The following enzymes in L. mexicana were tested for inhibition by formycin B (2 μM - 1 mM) with no signficant effect observed.

- adenine, guanine and hypoxanthine phosphoribosyltransferases
- Guanase
- Adenase
- Inosine, guanosine and adenosine nucleosidases

Preliminary studies have shown that allopurinolriboside and formycin B appear to be effective analogues against certain species of leishmania and trypanosomes. The biochemical mode of action of these compounds appears to be similar but to vary quantitatively. Our preliminary results suggest that the most critical action of these drugs occurs by their interaction with RNA, ribosomes and DNA.

Formycin B(FoB) is a structural analog of inosine that is a potent inhibitor of Leishmania multiplication. FoB is reportedly converted to formycin A nucleotides and formycin A-containing RNA by the organisms, and it is unclear whether the active form of the drug is the nucleoside itself or its several metabolites.

We have found that the uptake of FoB by Leishmania mexicana promastigotes was linear up to 4 hours, and resulted in a 6-fold concentration of FoB and its nucleotide metabolites within the organisms compared to the extracellular medium. The metabolites —FoB-MP, FoA-MP, FoA-DP, and FoA-TP--and FoA-containing RNA have previously been demonstrated in FoB exposed Leishmania (Rainey and Santi, 1983; Nelson et al, 1982; Berman et al, 1983). The possibility that FoB itself might inhibit parasite function led to the investigation of the effect of FoB on purine nucleoside uptake and purine base phosphoribosylation. Under the conditions employed, neither nucleoside transport nor base phosphoribosylation was significantly inhibited by FoB.

We demonstrated that FoB did profoundly decrease DNA, RNA, and protein synthesis as early as 1 hour after exposure of the organisms to the drug, and decreased synthesis of all three macromolecules at 24 hours by >90%. Because inhibition of protein synthesis could be a consequence of inhibited mRNA translation, we investigated whether translation was inhibited by the FoA nucleotides formed in FoB-exposed organisms. When either FoA-MP or FoA-TP was preincubated with heated control mRNA, translation was inhibited by 90%. Heating of mRNA is recommended (Iynedjian and Hanson, 1977) in translation studies to partially correct for folding of the messenger during its isolation. The above inhibition may be due to non-specific binding of the FoA nucleotides to the mRNA. When either FoA-MP or FoA-TP was preincubated with the reticulocyte lysate, translation of mRNA was inhibited by approximately 80%. It has recently been demonstrated in such lysates that exogenous AMP inhibits polypeptide chain elongation (Mosca, 1983), and that ATP is a necessary cofactor for maximum protein synthesis (Jackson, 1983). The close structural similarity of FoA-MP to AMP and FoA-TP to ATP suggests that the mechanisms by which FoA-MP and FoA-TP inhibited translation in these experiments could be inhibition of polypeptide chain elongation and inhibition of ATP utilization, respectively.

Inhibition of protein sythesis in FoB exposed organisms might also be due to difficulty in translation of mRNA made by the organisms. The demonstration that translation of mRNA from such cells was 67% inhibited compared to control mRNA suggests that one mechanism for the effect of FoB on Leishmania is inhibited translation of FoA-containing mRNA.

This is apparently the first report in which the mechanisms by which antileishmanial purine nucleotides inhibit protein synthesis have been elucidated. The data we have obtained supports the hypothesis that the FoA nucleotides and FoA-containing RNA formed in FoB-exposed organisms are at least partially responsible for the activity of the drug.

RESULTS DURING LAST YEAR.

Most of our time during the last year has centered on the partial purification of RNA and DNA polymerase for the purpose of determining how FoA nucleotides are incorporated into RNA and to determine if FoA nucleotides inhibit DNA polymerase.

A comparison of RNA and DNA polymerase of Leishmania to host cells would also help in the design of antileishmanial drugs.

We have had considerable difficulty in the isolation and the maintenance of stability of these two very important enzymes. We have worked out many of our difficulties with DNA polymerase, but we are still working out the best procedures for the isolation of RNA polymerase from L. mexicana WR #227.

For the RNA polymerase assay many methods were tried. We found the most success with a method adapted from Smith, Steven and Braun (1978) Eur. J. Biochem. 82, 309-320. This assay has a final volume of 0.2 ml and contains the following: 1mM dithiothretiol, 1mM $MnCl_2$, 5mM $MgCl_2$, 50mM Tris HCl (pH 8.0), 10% (v/v) Sigma glycerol, 100 µg/ml bovine serum albumin, 0.6 mM ATP, CTP, and GTP, 30 µg denatured DNA, and 0.57 µM [^{3}H] UTP (36 Ci/mmole ICN).

During nucleotide concentration studies ATP was omitted and the appropriate nucleotide (ATP, FoAMP or FoATP) was added to the assay. These types of experiments were performed to test how well ATP analogs served as a substrate for RNA polymerase. All samples were incubated at 30°C for 10 minutes. The reaction was stopped by adding 0.8 ml of 5% TCA + 20 mM $Na_4H_2P_2O_7$. Samples were then filtered on Whatman GF/C filters and washed twice with 2% TCA + 2 mM $Na_4H_2P_2O_7$. Filters are then counted in 7 ml of scintillation fluid.

We found more activity with denatured DNA than native DNA. Activity was decreased as much as 34.6% when using native DNA over denatured DNA.

RNA Polymerase Purification

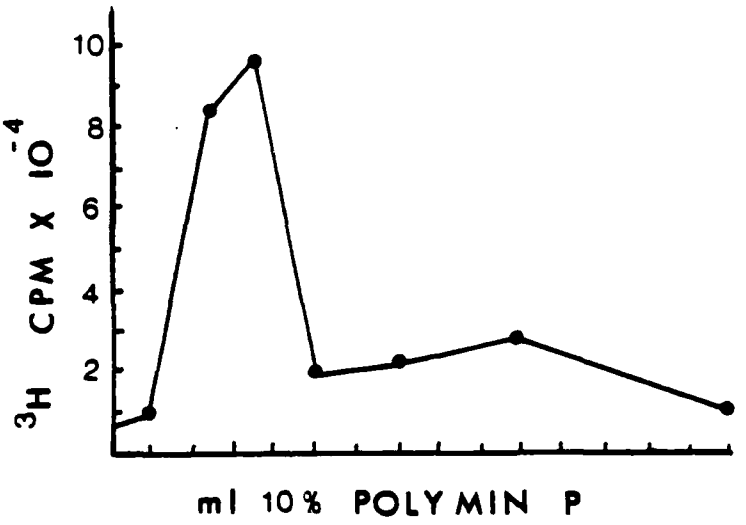
Our purification technique is a modification of that used by Jendrisak, and Burgess (1975) Biochem (14) 21, 4639-4645 and Smith and Braun (1978) Eur. J. Biochem. 82, 309-320.

Frozen (-70°C) L. mexicana 227 cells or freshly harvested cells are suspended in a 0.2M Tris HCl (pH 8.0), 50% (v/v) glycerol, 0.2M EDTA, 20 mM 2-mercaptoethanol and 1% Triton 100 solution. This suspension is sonicated and then centrifuged at 13,000 x g for 30 min. The supernatant is filtered through glass wool to remove lipid.

RNA polymerase and protein is then precipitated from the supernatant with Polymin P (Fig. 2). The indicated volumes of 10% Polymin P were added to 1 ml aliquots with thorough mixing. After centrifugation at 10,000 x g for 10 min, 5 µl aliquots of supernatnats were assayed for RNA polymerase. The optimal conditions for precipitating the RNA polymerase from the crude extract with

Fig. 2

Polymin P Precipitation of RNA Polymerase



Polymin P was determined from the curve. As seen in Fig. 2, RNA polymerase activity increases as inhibiting factors are precipitated from the extract. At a volume of 50 µl of Polymin P/ml extract, RNA polymerase itself is precipitated. RNA polymerase is reported to be released from the pellet by addition of $(NH_4)_2SO_4$, leaving most of the protein and nucleic acids in the pellet We have had problems with this step since the inhibiting substances also seem to be released. So, in order to start with a preparation with high activity we use the Polymin P supernatant to apply to columns. Fig. 3 shows the elution of RNA polymerase from DEAE-cellulose chromatography. This is a very efficient step resulting in a 15-fold purification with 78% recovery. High recovery during chromatographic steps requires the presence of high glycerol or ethylene glycol concentrations (25% v/v) in buffers. The latter agent was chosen because of its lower viscosity and density. A 1 ml sample of a Polymin P supernatant fraction was applied to a column (1.8 x 10 cm) of phosphocellulose in TEDG + 0.075 M $(NH_4)_2SO_4$. The sample was followed by 50 ml of TEDG + 0.075 M $(NH_4)_2SO_4$ to wash out unbound protein. RNA polymerase was then eluted either by a salt gradient or by a salt step. For salt gradient elution, RNA polymerase was eluted with a linear gradient of $(NH_4)_2SO_4$ from 0.075 to 0.25 M in 100 ml TEDG. Fig. 2 shows two peaks of RNA polymerase activity, the first much more active than the other.

Amaniten which is a known inhibitor of eucaryotic RNA polymerase II was added to the active fractions which were then assayed. The activity from Peak 1 was 62% inhibited by 1 µg/ml - amanitin. The activity from peak 1 was pooled and the protein concentrated by $(NH_4)_2SO_4$ precipitation. We have found the enzyme to be very unstable after this step, and we are currently trying to devise a method to stabilize the enzyme.

We have found the enzyme is destroyed by excessive sonication, but sonication is necessary to release RNA polymerase into the supernatant (Fig. 4).

We varied the $MgCl_2$ concentration in our reaction mixture to determine if we could enhance polymerase activity. Fig. 5 shows that increasing our concentration of $MgCl_2$ from 1 mM to 3 mM had a beneficial effect.

We varied the ATP concentration in our reactions and found that 0.6 mM ATP gave the optimal activity under the conditions used. To determine how efficiently FoATP and FoAMP were used as substrates by RNA polymerase, ATP was left out of the reaction mixture and FoA nucleotides were substituted at varies concentrations. Fig. 6 shows that FoATP is only 13% as efficient as ATP in serving as a substrate for RNA polymerase under the conditions tested. FoAMP appears not to be a substrate. When FoA nucleotides are added to an assay mixture along with ATP, we see slight stimulation to no effect.

Partial Purification of DNA Polymerase

We have partially pruified the DNA polymerase from L. mexicana, and have tested the effect of formycin A nucleotides on this enzyme. Cells were sonicated in 0.1M Tris-HCl, pH 7.8 buffer with 20% (v/v) glycerol and fractionated by centrifugation at 4° in a Sorvall SS-34 rotor at 10,000 x g for 1 h. The pellet was discarded, and the resulting supernatant fraction was made 1% (v/v) in respect to Triton X-100 and 0.4M in respect to KCl. After

Fig. 3

DEAE-Cellulose Chromatography of RNA Polymerase

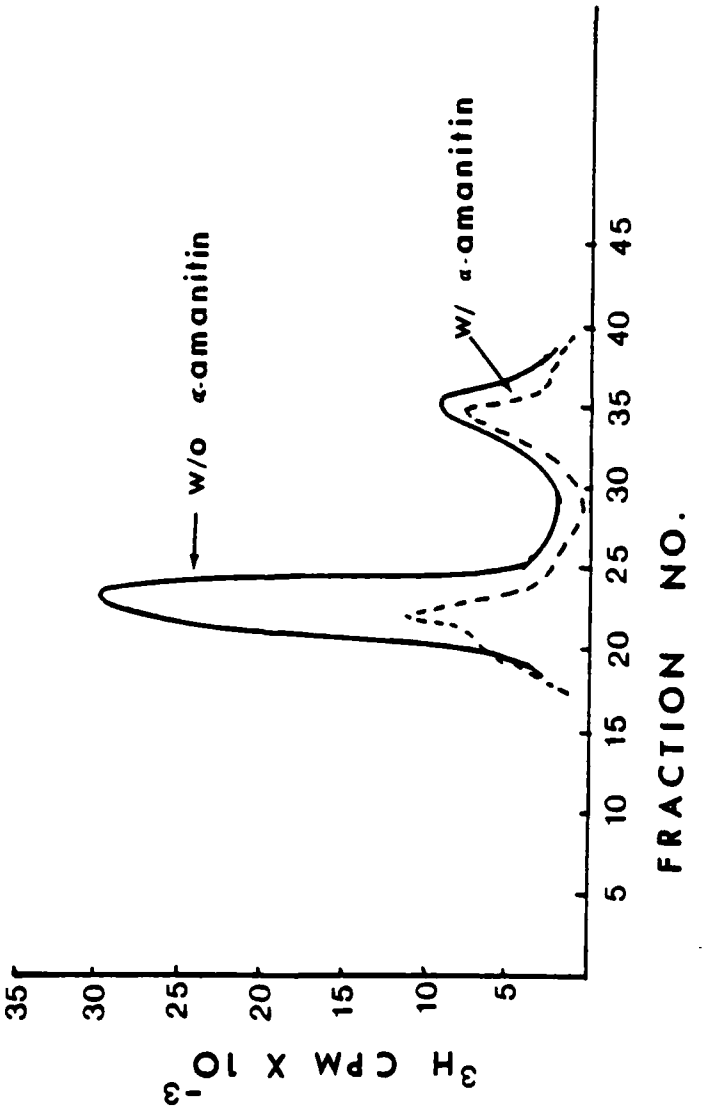


Fig. 4

The Effect of Sonication on RNA Polymerase Activity

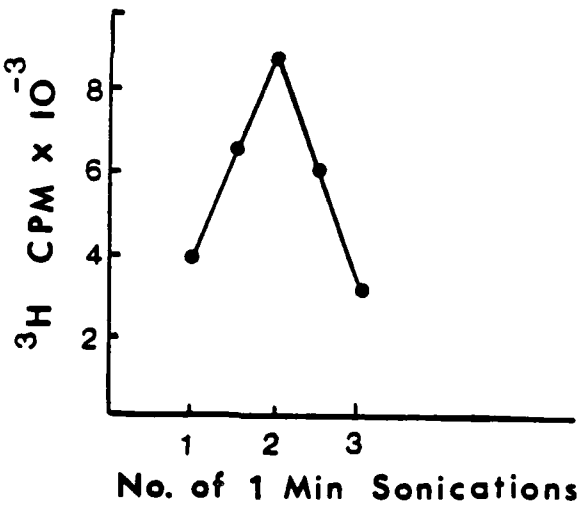


Fig. 5

RNA Polymerase Activity with Varying Concentrations of $MgCl_2$

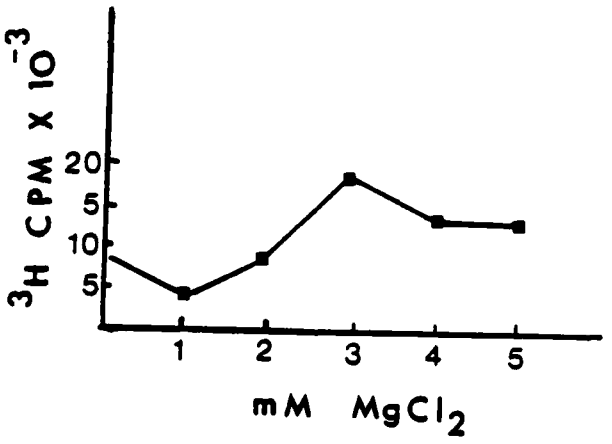
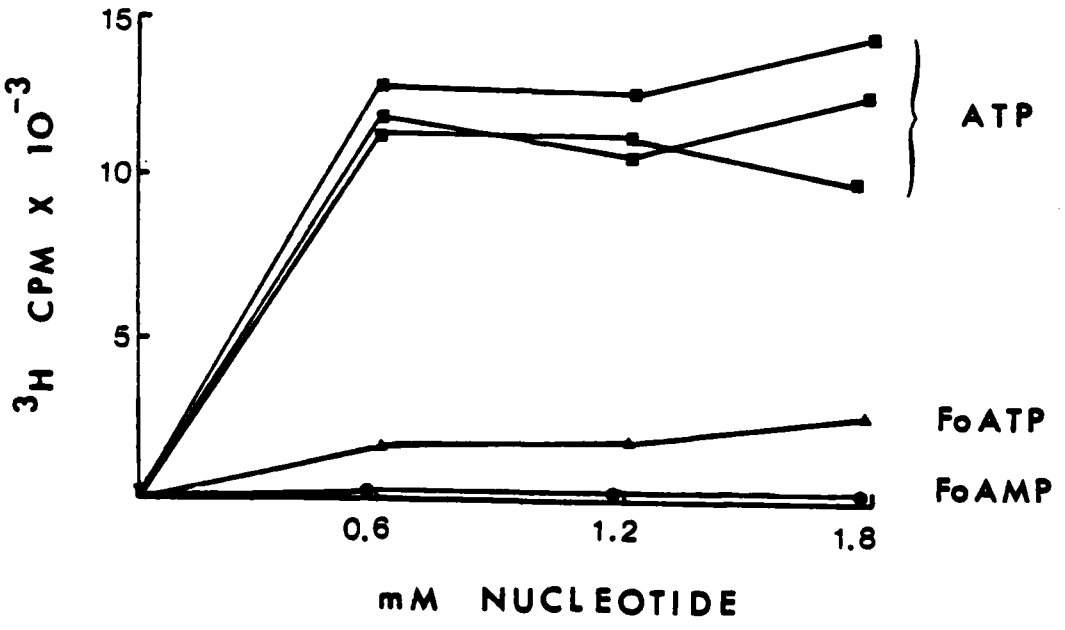


Fig. 6

The Ability of Different Nucleotides to Serve as a Substrate for RNA Polymerase



gentle mixing the solution was centrifuged in a Beckman SW41 rotor at 100,000 x g for 45 min. at 4°. The supernatant fraction was then passed through a DEAE-sephadex column equilibrated with buffer containing 0.05 M KCl, 1mM dithiothreitol, 0.4M KCl and 10% glycerol to remove any associated nucleic acids. The peak fractions were pooled, concentrated by ammonium sulfate, dialyzed and put on a phosphocellulose column equilibrated with 50mM KCl. The column was washed with 20 ml of this buffer containing 70mM KCl and eluted with a 200ml linear KCl gradient (0.1-0.6M KCl) in 40mM Tris-HCl, pH 7.5, 10% glycerol. Fig. 7 shows the DNA polymerase acitivity profile.

Initially we were using a radioactive assay technique involving filtration of filter pads, but the assay gave us inconsistant results with very high background counts. We are now using the non-radioactive assay technique developed by Sigma Chemical Co. This assay has proven very satisfactory.

Using the following purification steps we have increased the specific activity 16 fold and have gotten rid of 98% of the protein.

Preparation	Specific Activity units/mg	Total Protein
Lysed Cells	8.9	693.16
Crude Extract	39.97	414.54
Polymin P supernatant	22.60	109.78
$(NH_4)_2SO_4$ solobalisation of Polmin P pellett	165.36	6.72
DNA-Agarose peak	144.62	1.05

We have added FoA, FoB and their nucleotides at various concentrations with varying amounts of deoxynucleotides used for this assay, and we have found slight stimulation to no effect on this enzyme. Our conclusion is that under the conditions of testing formycin metabolites do not effect DNA polymerase to any significant extent.

We plan to use our purification procedure to test the effect of other purine analogs on this enzyme.

Uptake

UPTAKE STUDIES WITH PURINES AND PYRIMIDINES

Uptake studies involving a procedure which is based on rapid separation (5 sec) of medium from cells by centrifugation (Kidder, Dewey and Nolan, 1978, J. Cell. Physiol., 96, 165-170) were performed using L. mexicana WR 227. These studies were carried out using whole cells of the parasites along with labeled purine and pyrimidine bases and nucleosides. Incubations were carried out in 1.5 ml microcentrifuge tubes.

Fig. 7

DNA Polymerase Assay on Fractions from Phosphocellulose Column

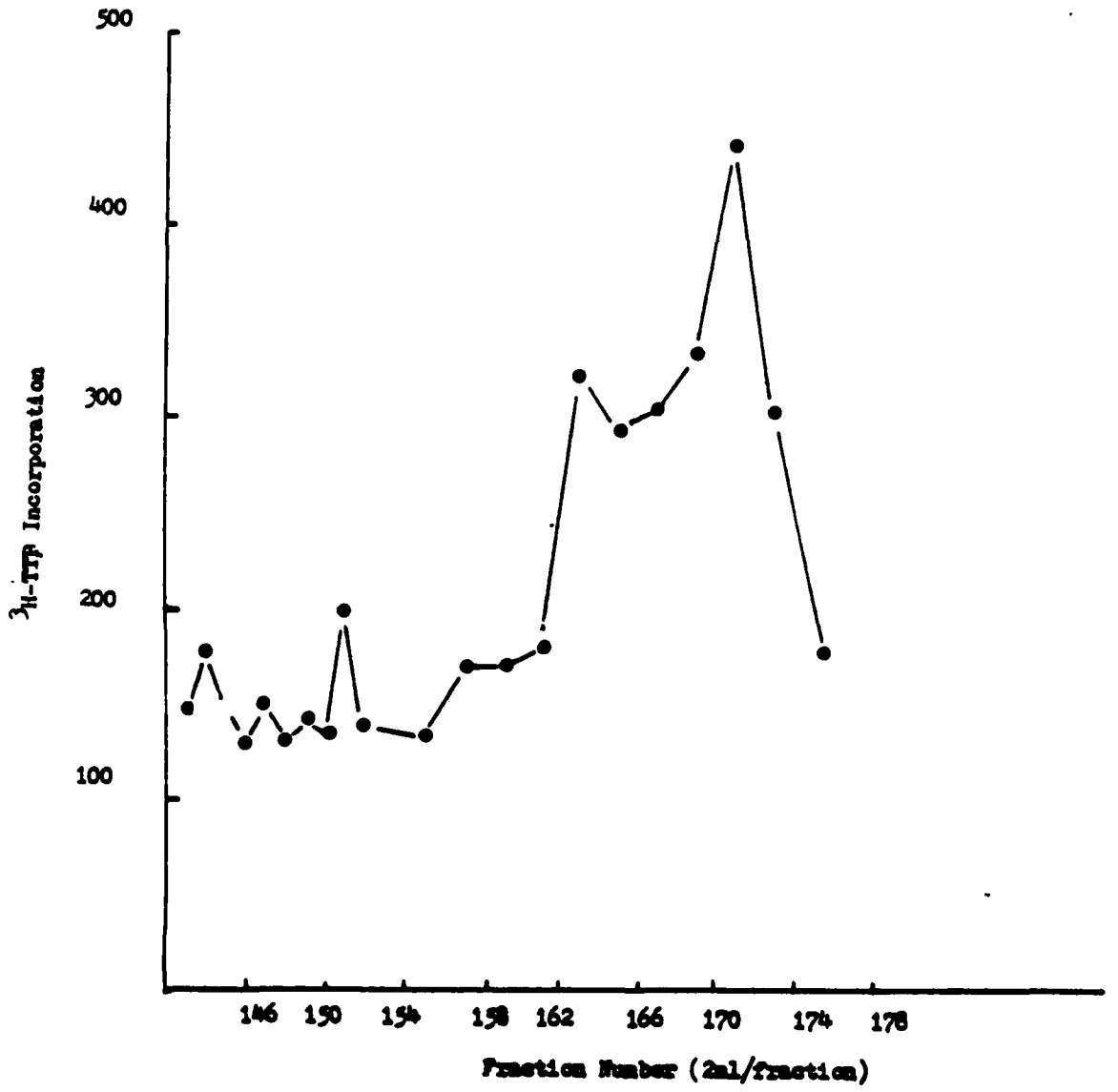


Fig. 8 shows uptake of adenine and adenosine as a function of pH. This data suggests that we are studying a carrier mediated transport system. If simple diffusion were involved, the pH would not have an effect.

Fig. 9 shows the appearance and dissappearance of transport capabilities with time of L. mexicana inoculated into a purine free medium (Steigher and Black). As shown these cells develop a much higher capability to transport adenosine with time during purine starvation as when compared to the other purines.

The scanning electron micrographs shown in Figure A, a normal 48 hr purine "starved" L. donovani cell and in Figure B a similar cell exposed to 5 mM caffeine (20 min). It has been determined with the use of C^{14} caffeine that caffeine does not accumulate in these cells and that the addition of 2mM adenosine will reverse the morphological effects of caffeine up to 3 hrs. 5mM caffeine will inhibit transport of adenosine by 35% in 2 min. If the caffeine exposed cells are washed free of caffeine the adenosine transport is restored as well as its shape. This data suggests that caffeine attaches to one of the adenosine receptor sites which inhibits adenosine transport. It also suggests that saturation of receptor sites on the surface alters cell shape.

In Vivo Growth Experiments with Cyclic FoA

We have found that cyclic FoA is as potent an inhibitor as FoA and FoB in L. mexicana 227 (Fig. 10). We plan to do more in vivo growth experiments and to investigate cFoA metabolism in Leishmania.

ISOLATION OF MESSENGER RNA

We are currently isolating and stock piling mRNA from purine analog treated cells and unexposed cells. We are doing this so that our experiments testing the results of different analogs will be done with the same reticulocyte lysate preparation and other stock solutions making the experiments as comparable as possible.

Fig. 8

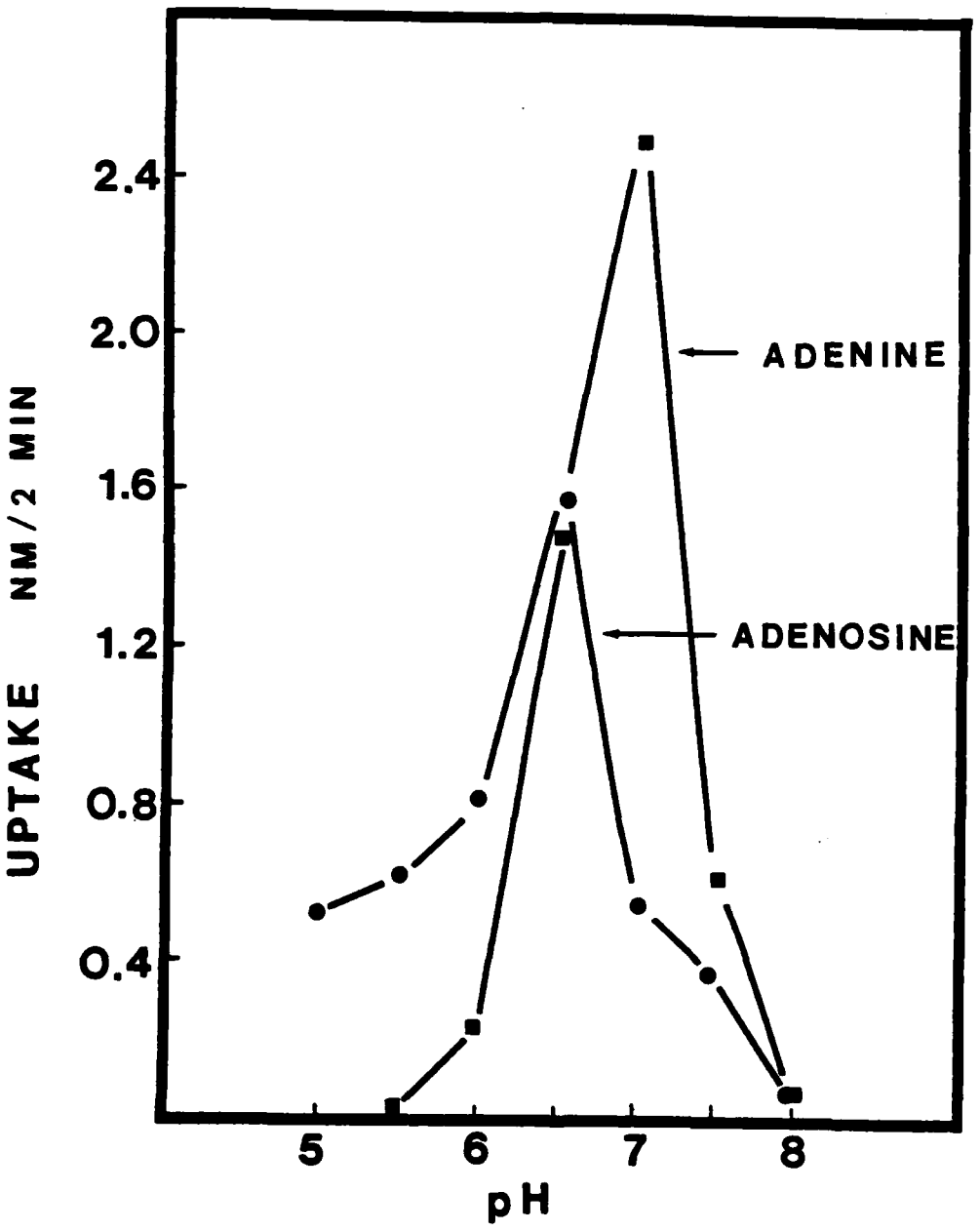
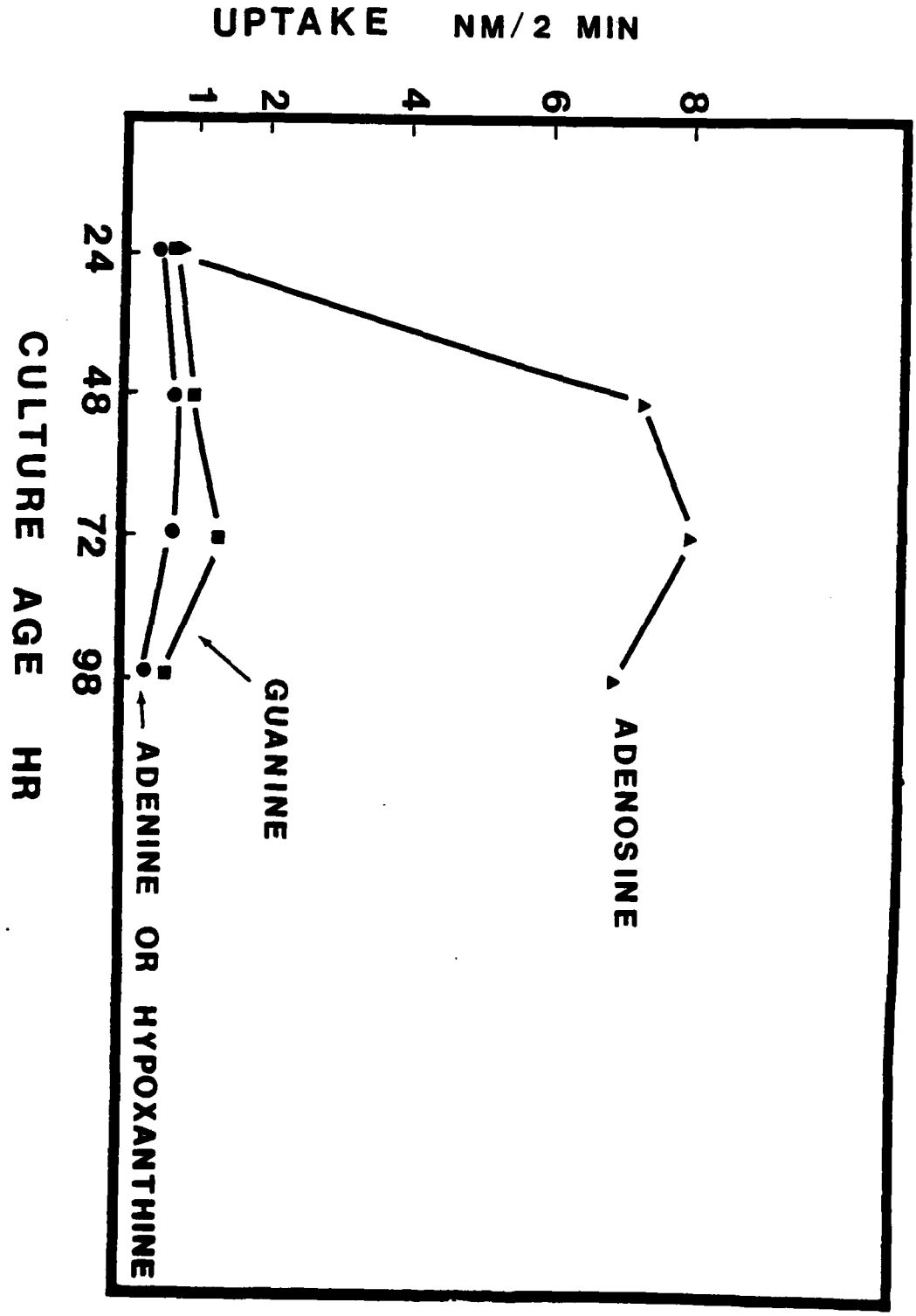


Fig. 9


UPTAKE NM/2 MIN
1
2
4
6
8
CULTURE AGE HR
24
48
72
98
ADENOSINE
GUANINE
ADENINE OR HYPOXANTHINE

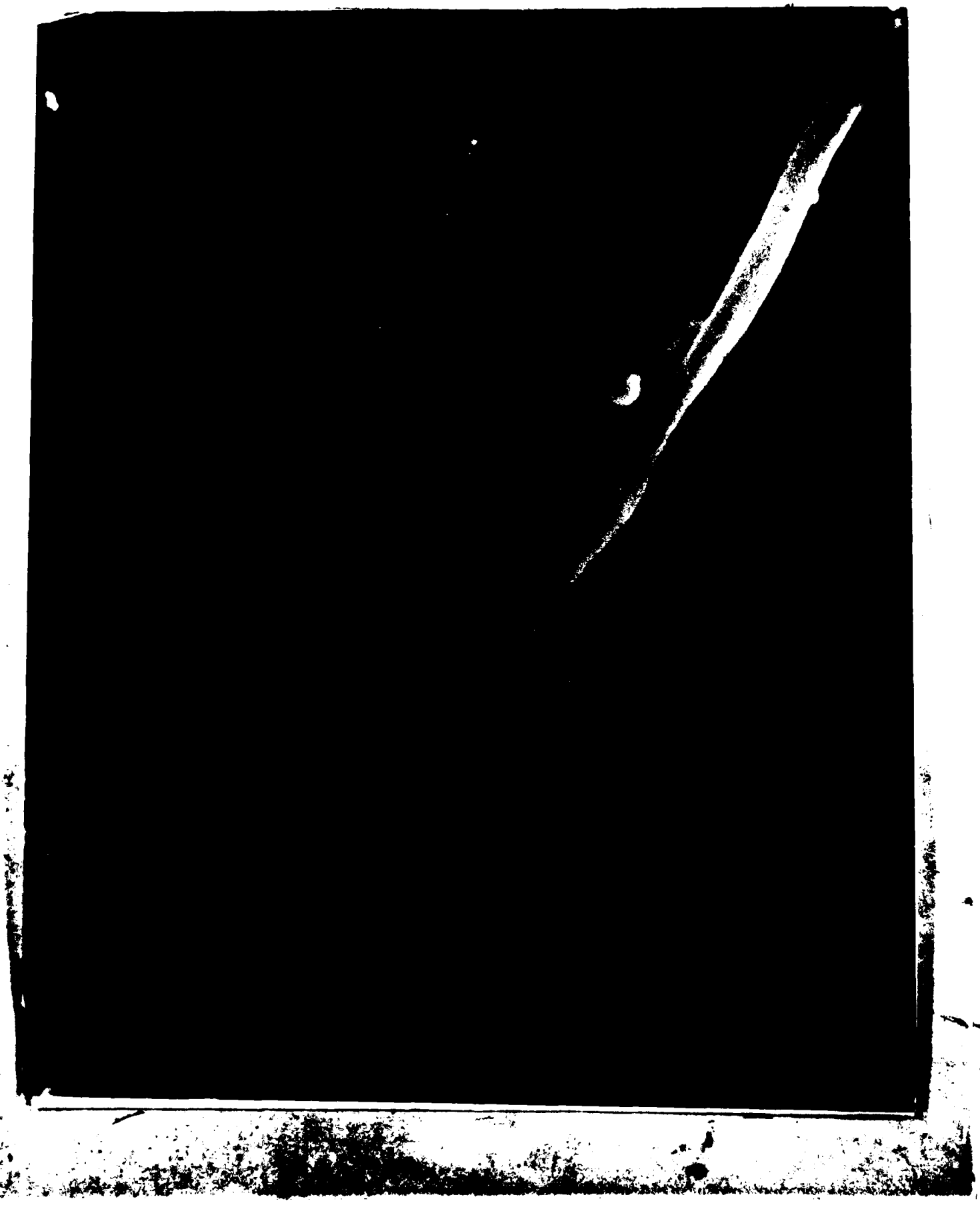
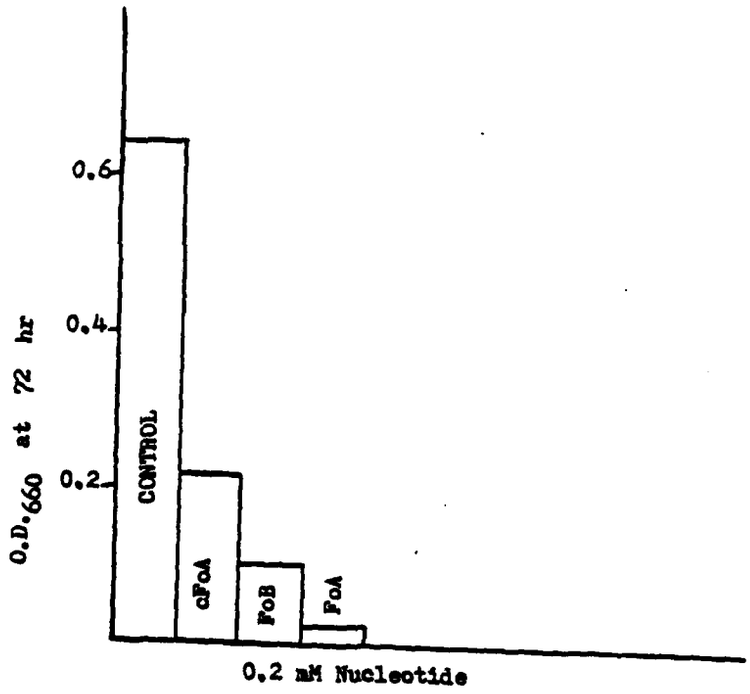


Figure A

Figure B

Fig. 10

Growth of L. mexicana in the Presence of Formycin Compounds



SPECIFIC AIMS AND MILITARY SIGNIFICANCE:

The need for leishmanicides cannot be overemphasized. At present chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality, presumably due to strain differences in Leishmania.

To date the logical design of antiparasitic drugs has proved largely unsuccessful with the exception of purine metabolism in protozoa. While mammalian cells are capable of de novo synthesis of purines, many parasites do not synthesize purines but use salvage pathways. Analogs inhibiting key enzymes in the salvage pathways should, therefore, provide novel therapeutic agents. Purines and pyrimidines serve not only as precursors of RNA and DNA, but also as stores of high energy phosphate, constituents of certain coenzymes, and modulators of various enzymatic reactions. In view of this vital role, intervention of their metabolism will have profound effects on the organism.

To date there is no safe, effective, and quality-controlled anti-parasitic vaccines. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult.

Parasite enzymes located on or near the surface of the membrane and transport and receptor proteins on the membrane which differ from the hosts are candidates as molecules which provoke host-protective immunity.

Leishmania and Trypanosoma studied have been found to require exogenous purine (Gutteridge, 1978) as opposed to the majority of mammalian cells. This fact together with the finding that some purine metabolizing enzymes are different (Kidder and Nolan 1979; Nolan and Kidder, 1980) may provide a useful target for immune intervention.

The elucidation of the biochemical mode of action of promising compounds (allopurinol riboside, formycin B) will permit the logical design of more effective derivatives and also will provide insight on the mechanism of drug resistance. This information may allow a therapy program to be developed which would decrease or eliminate the problem of drug resistance.

SPECIFIC AIMS

1) The mode of action of formycin B and its major metabolites, formycin A mono, di and triphosphate will be determined at the transcription and translation level. Their mode of action will be compared to allopurinol riboside and 4-aminopyrazolopyrimidine.

Altered structure of mRNA, ribosomes, tRNA and DNA will be determined by in vitro assay techniques and gel electrophoresis. RNA and DNA polymerase will be partially purified and formycin compounds will be tested for inhibition of their activity.

Once we determine the site or sites of action of formycin metabolites, if WRAIR wishes we will test formycin derivatives being synthesized by Dr. Roland K. Robins, Director of the Cancer Research Center, Provo, Utah. Dr. Robins is also under contract to WRAIR. Techniques which will be employed are described in:
1). Clemens, et al., J. Biol. Chem. 250, 2 (1975) 522-526;
2). Methods in Enzymology, Vols. XX, XXI, XXII. These volumes describe techniques for nucleic acid and protein synthesis studies and isolation of RNA and DNA polymerases.

Our preliminary data suggests the possibility that formycin nucleotides may bind to ribosomes since incubation of formycin nucleotides with rabbit reticulocyte lysate before initiation of translation inhibits in vitro protein synthesis by 90% (see Previous Work on This Project). A number of very successful antibiotics (puromycin, Streptomycin, aminoglycosides, neomycin, kanamycin, gentamicin, hygromycin B, auritricarboxylic acid, pactamycin, edine, tetracylines, emetine, cryptopleurine, chloroamphenicol, erythromycin, lincomycin and Streptogramins, to name a few) have been shown to exert their toxic effects on ribosomes. We feel that ribosome characterization in Leishmania may prove very important in the elucidation of the mode of action of formycin B. Also, investigations of leishmanial ribosomes and their binding capacities may help in the design of a more effective drug.

We will isolate ribosomes from Leishmania and expose them to [^{3}H]-FoATP and [^{3}H]-FoAMP. These radioactive nucleotides will be synthesized from [^{3}H]-FoB by incubating Leishmania or red blood cells with [^{3}H-FoB and isolating the products by HPLC. The radioactive nucleotides will be incubated with ribosomal subunits which will then be collected (e.g. on Millipore filters), washed free from unbound nucleotide and assayed for radioactivity. This technique is adequate to detect tight binding but may give negative results with compounds which interact only weakly with ribosomes.

Weak binding can be tested using unlabelled FoA nucleotides and incubating them with ribosomal subunits. These subunits will then be combined with untreated partners to ascertain whether the nucleotide inactivates either subunit. These types of experiments have been used to demonstrate the mode of action of the antibiotics that were previously listed.

We will investigate further the incorporation of FoA nucleotides into total RNA. Total RNA of organisms treated or untreated with [^{3}H]-FoB will be isolated and fractionated by HPLC to rRNA, tRNA and mRNA. By this method we will be able to determine where most of the [^{3}H]-FoB is incorporated. Dr. Lallan Giri, at Pharmacia Fine Chemical Co., will help us in developing RNA isolation by HPLC.

After we determine the site (s) of action of FoA nucleotides in Leishmania, we will use dog liver to determine the metabolism of [^{3}H]-FoB and test its effects on liver protein synthesis. Berman (personal communication) has reported that FoB administration to dogs has produced liver damage. Detailing the metabolism and mode of action of FoB in dog liver will allow one to either circumvent its metabolism in the liver or produce a more specific analog for treatment of leishmaniasis. For example, if FoB is phosphorylated by adenonine kinase in the liver and by nucleoside phosphotransferase in Leishmania, an inhibitor of adenosine kinase would stop FoB metabolism and prevent its toxic effects in liver. Also, one might synthesize an analog which would not serve as a substrate for adenosine kinase, but would for nucleoside phosphotransferase and still maintain the same major mode of action of FoB.

Purine analog metabolism in liver is important since the liver is believed to be a major regulator of purine concentration in plasma (Arnold and Cysyk, 1983). These authors found that in rat liver the purines hypoxanthine, inosine, and adenine were all found to be completely cleared in a single passage, but differed in their metabolic fate after uptake. Studies by several groups have provided evidence that rapidly proliferating cells within several different organs, such as intestinal epithelium, bone marrow and spleen, use purine compounds as supplemental nutrients. They are provided by the liver and carried to the organs by erythrocytes (Lerner and Lowy, 1974; Lowy and Lerner, 1974; Pritchard et al. 1970).

(2) The in vivo and in vitro metabolism of cyclic formycin A (cFoA) will be investigated by HPLC chromotography.

We have found that when L. mexicana cells are exposed to [^{3}H]-FoB and the cell extract analyzed by HPLC, a peak which co-elutes with authentic cFoA is present. Also, in vivo growth experiments with L. mexicana have shown that cFoA is as inhibitory as FoA and FoB (see section, Previous Work on This Project).

A correlation between cyclic AMP concentration within Leishmania cells and proliferation and transformation has been demonstrated. In the case of L. donovani the transformation of amastigotes to promastigotes was inhibited by the addition of dibutyryl cAMP (Walter and Ebert, 1978).

We will determine if cFoA is inhibitory in Leishmania due to (1) its metabolism to FoA nucleotides or (2) its action as an cAMP analog or (3) both 1 and 2.

(3) We will continue to study purine and pyrimidine uptake and accumulation in the presence and absence of promising analogs. We will also study this area of metabolism in promastigote to amastigote transformation. Initially a revision of the media of Pan (1984) will be used for this investigation. We will compare the results obtained from amastigotes grown in cell-free medium to those isolated from cell line J774. Looker, Berens and Marr (1983) have reported that adenine deaminase is present in promastigotes of L. donovani but absent in amastigotes with the opposite situation occurring for the presence

of adenosine deaminase. Alterations of the purine concentrations in a medium for amastigotes will allow us to determine if the cellular environment induces adenosine deaminase production or if the production of adenosine deaminase is dependent on the transformation process.

A great amount of attention will be placed on adenosine metabolism, since we have obtained preliminary data suggesting that during purine starvation L. mexicana develops the capacity to accumulate adenosine more than any other purine tested (see section, Previous Work on This Project, 1983). Also, we have obtained evidence that compounds which attach to adenosine receptors alter cell shape.

Many biological properties of adenosine have been identified: it is toxic to mammalian and bacterial cells, and its presence is associated with inhibition of the immune response, coronary dilation, delayed neurotransmission, inhibition or stimulation of hormone secretion, and changes in the metabolism of a number of tissues. Adenosine has many biochemical effects including: direct activation of adenylate cyclase, inhibition of pyrimidine biosynthesis and diminution of phosphoribosyl-pyrophosphate (Fox and Kelley, 1978).

Investigations of the possible role adenosine may play in parasite transformation and/or alteration of macrophage function will help in the development of antileishmanial compounds and understanding the disease process.

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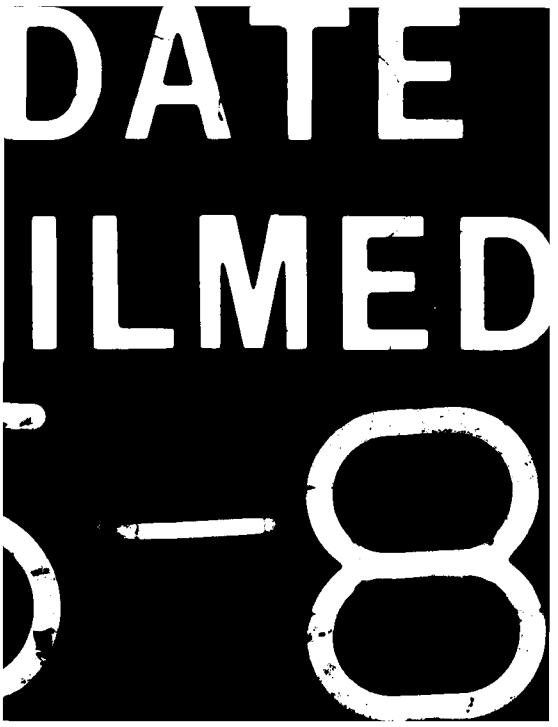
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